

TITLE OF INVENTIONRECOMBINANT HIGH MOLECULAR WEIGHT MAJOR OUTER  
MEMBRANE PROTEIN OF MORAXELLAREFERENCE TO RELATED APPLICATION

5           This application is a continuation-in-part of copending United States Patent Application No. 09/361,619 filed July 27, 1999.

FIELD OF INVENTION

          The present invention relates to the field of immunology and is particularly concerned with outer membrane proteins from *Moraxella*, methods of  
10 recombinant production thereof, genes encoding such proteins and uses thereof.

BACKGROUND OF THE INVENTION

          Otitis media is the most common illness of early childhood with approximately 70% of all children suffering at least one bout of otitis media before the age of seven. Chronic otitis media can lead to hearing, speech and cognitive  
15 impairment in children. It is caused by bacterial infection with *Streptococcus pneumoniae* (approximately 50%), non-typable *Haemophilus influenzae* (approximately 30%) and *Moraxella (Branhamella) catarrhalis* (approximately 20%). In the United States alone, treatment of otitis media costs between one and two billion dollars per year for antibiotics and surgical procedures, such as  
20 tonsillectomies, adenoidectomies and insertion of tympanostomy tubes. Because otitis media occurs at a time in life when language skills are developing at a rapid pace, developmental disabilities specifically related to learning and auditory perception have been documented in youngsters with frequent otitis media.

*M. catarrhalis* mainly colonizes the respiratory tract and is predominantly a  
25 mucosal pathogen. Studies using cultures of middle ear fluid obtained by tympanocentesis have shown that *M. catarrhalis* causes approximately 20% of cases of otitis media (ref. 1 - Throughout this application, various references are referred to in parenthesis to more fully describe the state of the art to which this invention pertains. Full bibliographic information for each citation is found at the end of the

specification, immediately preceding the claims. The disclosures of these references are hereby incorporated by reference into the present disclosure).

The incidence of otitis media caused by *M. catarrhalis* is increasing. As ways of preventing otitis media caused by pneumococcus and non-typable *H. influenzae* are developed, the relative importance of *M. catarrhalis* as a cause of  
5 otitis media can be expected to further increase.

*M. catarrhalis* is also an important cause of lower respiratory tract infections in adults, particularly in the setting of chronic bronchitis and emphysema (refs. 2, 3, 4, 5, 6, 7, and 8). *M. catarrhalis* also causes sinusitis in children and adults (refs. 9,  
10 10, 11, 12, and 13) and occasionally causes invasive disease (refs. 14, 15, 16, 17, 18, and 19).

Like other Gram-negative bacteria, the outer membrane of *M. catarrhalis* consists of phospholipids, lipopolysaccharide (LPS), and outer membrane proteins (OMPs). Eight of the *M. catarrhalis* OMPs have been identified as major  
15 components. These are designated by letters A to H, beginning with OMP A which has a molecular mass of 98 kDa to OMP H which has a molecular mass of 21 kDa (ref. 20).

Recently, Klingman and Murphy purified and characterized a high molecular-weight outer membrane protein of *M. catarrhalis* (ref. 21). The apparent  
20 molecular mass of this protein varies from 350 kDa to 720 kDa as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). This protein appears to be an oligomer of much smaller proteins or subunits thereof of molecular mass about 120 to 140 kDa and is antigenically conserved among strains of *Moraxella*.

25 Helminen et al also identified a protein of molecular mass of about 300 to 400 kDa, named UspA, that was reported to be present on the surface of *Moraxella* (ref. 22).

In WO 96/34960 and US Patent No. 5,808,024, assigned to the assignee hereof and the disclosure of which is incorporated herein by reference, there is  
30 described a new protein of *M. catarrhalis* which had an apparent molecular mass of about 200 kDa. Western blot analysis using antiserum raised against the 200 kDa protein suggested that this protein was different from the large UspA protein (> 300

kDa), reported by the two groups in refs. 21 and 22. Recently, the gene sequences encoding two related proteins, UspA1 and UspA2, have been published (ref. 23). A sequence comparison between the two genes encoding the UspA proteins and the gene encoding the 200 kDa protein confirmed that the 200 kDa protein is different  
5 from either of the UspA1 and UspA2 proteins.

Fitzgerald et al (ref. 29) have identified a 200 kDa protein associated with haemagglutination. Transmission electron microscopy studies (ref. 30) showed that the 200 kDa protein associated with haemagglutination is present on the outer fibrillar layer of *M. catarrhalis*. Recently, a non-clumping variant of strain 4223 was  
10 prepared by serial passaging and it was observed that the non-clumping variant had reduced expression of both UspA and a 200 kDa protein that is not UspA (ref. 31). It is possible that this 200 kDa protein is the same as that described in WO 96/34960 and herein.

The 200 kDa protein described herein has been detected in most, but not all,  
15 strains of *Moraxella catarrhalis*, which have been isolated from various sources, including otitis media (OM), sputum, nasopharynx, expectorate and bronchial secretions. Table 1A below contains a listing of *M. catarrhalis* strains tested, their source and whether or not the 200 kDa protein is expressed.

In this application, the term "about 200 kDa protein" refers, as recited in the  
20 aforementioned USP 5,808,024, to family of outer membrane protein of *M. catarrhalis* having a molecular mass between about 160 and 230 kDa, as determined SDS-PAGE at 12% polyacrylamide following the procedure of Laemli, and includes proteins having varieties in their amino acid sequences including those naturally occurring in various strains of *Moraxella*.

25 *M. catarrhalis* infection may lead to serious disease. It would be advantageous to provide recombinant means for providing large quantities of 200 kDa outer membrane protein of *M. catarrhalis* strains and genes encoding such proteins from various *M. catarrhalis* strains for use as antigens in immunogenic preparations including vaccines, carriers for other antigens and immunogens and the  
30 generation of diagnostic reagents.

### SUMMARY OF THE INVENTION

The present invention is directed towards the provision of a recombinantly-produced purified and isolated outer membrane protein of *Moraxella catarrhalis* and other *Moraxella* strains, having an apparent molecular mass of about 200 kDa, as well as genes encoding the same from various strains of *Moraxella catarrhalis*.

In one aspect of the present invention, there is provided an isolated and purified nucleic acid molecule having (a) a nucleotide sequence set forth in Figure 3, 4 or 5 (SEQ ID Nos: 5, 6, 8, 10) for *Moraxella catarrhalis* strains 4223, Q8 and LES-1 respectively or the complementary sequence thereto; (b) a nucleotide sequence encoding an about 200 kDa outer membrane protein of a strain of *Moraxella catarrhalis* and having the derived amino acid sequence shown in Figures 3, 4 or 5 (SEQ ID Nos: 7, 9, 11) for *Moraxella catarrhalis* strains 4223, Q8 and LES-1 respectively; and (c) a nucleotide sequence encoding an about 200 kDa outer membrane protein of another strain of *Moraxella catarrhalis* which is characterized by a tract of consecutive G nucleotides which is 3 or a multiple thereof in length, an ATG start codon about 80 to 90 bp upstream of said tract and said tract being located between about amino acids 25 and 35 encoded by the nucleotide sequence.

The another strain of *Moraxella catarrhalis* in (c) is a strain as identified in Table 1A other than strains 4223, Q8 and LES-1 and expressing an about 200 kDa protein.

In another aspect of the invention, there is provided an isolated and purified nucleic acid molecule having a nucleotide sequence selected from the group consisting of (a) a nucleotide sequence set forth in Figure 8 (SEQ ID No: 12) for a 5'-truncation of the gene encoding an about 200 kDa outer membrane protein of *Moraxella catarrhalis* strain 4223 contained in pKS348; (b) a nucleotide sequence encoding the derived amino acid sequence set forth in Figure 9 (SEQ ID No: 13) for a N-terminal truncation of an about 200 kDa outer membrane protein of *Moraxella catarrhalis* strain 4223 produced by pKS348; (c) a nucleotide sequence set forth in Figure 21 (SEQ ID No: 45) for a 5' truncation of the gene encoding an about 200 kDa outer membrane protein of *Moraxella catarrhalis* strain 4223 contained in pQWF; (d) a nucleotide sequence encoding the derived amino acid sequence set forth in Figure 21 (SEQ ID No: 46) for a N-terminal truncation of an about 200 kDa

outer membrane protein of *Moraxella catarrhalis* strain 4223 produced by pQWF; (e) a nucleotide sequence set forth in Figure 21 (SEQ ID No: 47) for a 5'- and 3'-truncation of the gene encoding an about 200 kDa outer membrane protein of *Moraxella catarrhalis* strain 4223 contained in pBR T7 3' 200 kDa(t); (f) a  
5 nucleotide sequence encoding the derived amino acid sequence set forth in Figure 21 (SEQ ID No: 48) for a N-terminal and C-terminal truncation of an about 200 kDa outer membrane protein of *Moraxella catarrhalis* strain 4223 produced by pBR T7 3' 200 kDa(t)/*KanR/cer*; (g) a nucleotide sequence encoding a 5'-truncation of a gene encoding an about 200 kDa outer membrane protein of another strain of *Moraxella*  
10 *catarrhalis* corresponding to those of (a), (b), (c) and (d) and being capable of expressing the corresponding N-terminally truncated about 200 kDa outer membrane protein from *E. coli*; and (h) a nucleotide sequence encoding a 5'- and 3'-truncation of a gene encoding an about 200 kDa outer membrane protein of another strain of *Moraxella catarrhalis* corresponding to those of (e) and (f) and being  
15 capable of expressing the corresponding N- and C-terminally truncated about 200 kDa outer membrane protein from *E. coli*.

A further aspect of the invention providing an isolated and purified nucleic acid molecule which is a contiguous *Nde* I – *Pst* I fragment of SEQ ID No: 5.

The invention, in an additional aspect, provides a vector for transforming a  
20 host comprising a nucleic acid molecule as provided herein, which may be a plasmid vector. The plasmid vector may be one which has the identifying characteristics of pKS348 (ATCC 203,529) or pKS294 (ATCC 203,528). The plasmid vector also may be one having the identifying characteristics of pQWF. In addition, the plasmid vector may be one having the identifying characteristics of pBR pT7 3' 200 kDa(t)  
25 pBR T7 3' 200 kDa(t)/*KanR* or pBR T7 3' 200 kDa(t)/*KanR/cer*.

A further aspect of the invention provides a host cell, such as *E. coli*, transformed by a vector provided herein and expressing an about 200 kDa protein of a strain of *Moraxella catarrhalis* or a truncation thereof. The invention further provides, in an additional aspect, a recombinant about 200 kDa outer membrane  
30 protein of a strain of *Moraxella catarrhalis* or a truncation thereof producible by the transformed host provided herein.

As set forth below, various truncated forms of the full length recombinant about 200 kDa protein may be produced. In one such truncation, the protein is truncated at the N-terminal end by reason of a short deletion to amino acid 56, produced by pKS348. As set forth below, the full-length protein appeared to be  
5 toxic to *E. coli* when expressed from pKS294, and only when truncated was the protein expressed.

The truncations further include the approximately C-terminal half of the protein, which appeared to be conserved among strains of *Moraxella catarrhalis* and, when expressed from pQWF, was produced in significantly larger quantities  
10 than from pKS348. Plasmid pQWF expressed the truncated protein as a doublet, the higher molecular weight band corresponding to the C-terminal half of the protein and the lower molecular weight band being a C-terminal truncation. The encoding nucleic acid and amino acid sequence of the C-terminal truncation are identified herein, expression vectors containing the nucleic acid are constructed, and the C-  
15 terminal truncation expressed.

The present invention includes nucleic acid molecules encoding portion only of the about 200 kDa protein of a strain of *M. catarrhalis* which are capable of being expressed as a truncated form of the about 200 kDa protein and the corresponding truncated form of the protein.

20 The recombinant about 200 kDa outer membrane protein or a truncation thereof may be formulated into an immunogenic composition, which may be formulated as a vaccine for *in vivo* administration to protect against disease caused by *Moraxella catarrhalis*, which may be provided in combination with a targeting molecule for delivery to specific cells of the immune system, formulated as a  
25 microparticle, capsule or liposome preparation, and may further comprise an adjuvant.

The invention, in a further aspect, includes a method of inducing protection against disease caused by *Moraxella catarrhalis* by administering to a susceptible host, which may be a human, an effective amount of the immunogenic composition  
30 provided herein.

In an additional aspect, the invention provides a method for the production of an about 200 kDa outer membrane protein of a strain of *Moraxella catarrhalis* or truncation thereof, which comprises:

- transforming a host cell, such as *E. coli*, with a vector as provided herein,
- 5 growing the host cell to express the encoded about 200 kDa protein or truncation thereof, and
- isolating and purifying the expressed about 200 kDa protein or truncation thereof.

The encoded about 200 kDa protein or truncation thereof may be expressed  
10 in inclusion bodies. The isolation and purification of the about 200 kDa protein may be effected by:

- disrupting the grown transformed cells to produce supernatant and the inclusion bodies,
- solubilizing the inclusion bodies to produce a solution of the recombinant  
15 about 200 kDa protein or truncation thereof,
- chromatographically purifying the solution of recombinant about 200 kDa protein or truncation thereof free from contaminating proteins, and
- isolating the purified recombinant about 200 kDa protein or truncation thereof.

20 Advantages of the present invention include:

- identification of nucleic acid and derived amino acid sequences of about 200 kDa protein from various strains of *M. catarrhalis*.
- production of immunogenic recombinant about 200 kDa protein, which may be in various truncated forms.

25 BRIEF DESCRIPTION OF DRAWINGS

Reference is made herein to the accompanying drawings, wherein:

Figure 1 shows restriction maps of subclones of a gene encoding the 200 kDa outer membrane protein of *M. catarrhalis* from  $\lambda$ EMBL3 clone 8II and the location of PCR primers used to amplify the 5'-region of the gene. The open reading  
30 frame of the about 200 kDa outer membrane protein is indicated by the shaded box. The numbers in parenthesis are approximate sizes of DNA inserts in plasmids.

Restrictions sites are Sal: *SaII*, N: *NcoI*, B: *BglII*, K: *KpnI*, Xb: *XbaI*, Xh: *XhoI*, RV: *EcoRV*;

Figure 2 shows the nucleotide sequence (SEQ ID No: 1 - entire sequence, SEQ ID No: 2 - coding sequence) of the gene encoding the about 200 kDa outer  
5 membrane protein of *M. catarrhalis* strain 4223, as determined from  $\lambda$ EMBL3 clone 8II, and deduced amino acid sequence (SEQ ID No: 3 - identified GTG start codon, SEQ ID No: 4 - putative ATG start codon shaded) of the about 200 kDa outer membrane protein. A ten-G nucleotide segment of the 5'-UTR is identified by underlining. An ATG start codon for the same sequence but with a nine-G  
10 nucleotide segment is identified by a box (see Figure 3);

Figure 3 shows the nucleotide sequence (SEQ ID No: 5 - entire sequence, SEQ ID No: 6 - coding sequence) of the gene encoding the about 200 kDa outer membrane protein of *M. catarrhalis* strain 4223, as determined from PCR-amplified genomic DNA of strain 4223 and the deduced amino acid sequence (SEQ ID No: 7)  
15 of the corresponding about 200 kDa outer membrane protein. A nine-G nucleotide segment of the sequence corresponding to the 10-G nucleotide segment of Figure 2, is identified by underlining. The GTG start codon identified in Figure 2 is identified by a box;

Figure 4 shows the nucleotide sequence (SEQ ID No: 8) of the gene  
20 encoding the about 200 kDa outer membrane protein of *M. catarrhalis* strain Q8 and the deduced amino acid sequence (SEQ ID No: 9) of the corresponding about 200 kDa outer membrane protein. A nine-G nucleotide segment is identified by underlining;

Figure 5 shows the nucleotide sequence (SEQ ID No: 10) of the gene  
25 encoding the about 200 kDa outer membrane protein of *M. catarrhalis* strain LES-1 and the deduced amino acid sequence (SEQ ID No: 11) of the corresponding about 200 kDa outer membrane protein. A three-G nucleotide segment is identified by underlining;

Figure 6 contains an alignment of the amino acid sequences (in single letter  
30 code) of the about 200 kDa proteins of *M. catarrhalis* strain 4223 (SEQ ID No: 7), Q8 (SEQ ID No: 9) and LES-1 (SEQ ID No: 11). The alignments of the sequences were made using BLAST and manual methods and are compared to the 4223



sequence. Gaps in the sequence where no corresponding or related amino acid exists are designated by "-" while identical amino acids are designed by ".";

Figure 7 shows the restriction sites of the *M. catarrhalis* strain 4223 derived 200 kDa protein gene as well as the identity of various plasmids containing partial or full length 200 kDa genes. Restriction sites are Sal: *SalI*, N: *NcoI*, K: *KpnI*, Xh: *XhoI*, Rv: *EcoRV*, Ps: *PstI*, Nd: *NdeI*;

Figure 8 shows the nucleotide sequence (SEQ ID No: 12) and deduced amino acid sequence (SEQ ID No: 13) of the 5'-truncated gene encoding the M56 200 kDa protein of *M. catarrhalis* strain 4223 contained in pKS348;

Figures 9A and 9B contain a schematic of the procedure for producing plasmid pKS294 expressing the full length 200 kDa protein of *M. catarrhalis* strain 4223;

Figure 10 is a schematic of the procedure for producing plasmid pKS348 expressing the N-truncated M56 r200 kDa protein of *M. catarrhalis* strain 4223;

Figure 11 shows a schematic procedure for the purification of recombinantly-produced 200 kDa protein from *E. coli*;

Figure 12 shows SDS-PAGE analysis of the expression of M56 r200 kDa protein gene from *E. coli*. *M. catarrhalis* strain 4223 lysate was run as a positive control (a) and uninduced KS358 cultured overnight was run as a negative control (b). In each lane, 20 µg of total protein was loaded;

Figure 13 shows the SDS-PAGE analysis of the purification of the M56 r200 kDa protein according to the scheme of Figure 11. Lane 1, *E. coli* whole cells; Lane 2, soluble proteins after 50 mM Tris/NaCl, pH8, extraction; Lane 3, soluble proteins after Tris/Triton X-100/EDTA extraction; Lane 4, soluble proteins after Tris/OG extraction; Lane 5, pellet after Tris/OG extraction; Lanes 6, 7, purified 200 kDa protein;

Figure 14 shows the anti-M56 r200 kDa protein antibody titers obtained in mice. Mice were immunized on day 1, day 29 and day 43 with 0.3 µg, 1 µg, 3 µg or 10 µg of the purified M56 r200 kDa protein in adjuvant. Antisera were obtained on days 14, 28, 42 and 56 and anti-M56 r200 kDa protein IgG titers were determined. The reactive titers of antisera were defined as the reciprocal of the dilution

consistently showing a two-fold increasing in absorbance over that obtained with the pre-bleed serum sample collected on day 0;

Figure 15 shows the anti-M56 r200 kDa antibody titers in guinea pigs. Guinea pigs were immunized and antisera were analyzed according to the protocol of Figure 14;

Figure 16 shows the location of PCR primers used to amplify a DNA fragments carrying portions of the 200 kDa protein gene from chromosomal DNA of *M. catarrhalis* strain RH408, a spontaneous mutant of strain 4223 which does not produce the 200 kDa protein;

Figure 17 is a partial nucleotide and derived amino acid sequence for the 200 kDa protein of *M. catarrhalis* strain 4223, indicating by arrows the locations of the initial amino acid of the respective three truncations ALA<sup>12</sup>, VAL<sup>19</sup> and GLY<sup>39</sup>;

Figure 18 shows schematic diagrams for two 3' half clones of the 4223 200 kDa gene. Clone pQWE contains a fusion between the 5' end of the 200 kDa gene and the 3' half of the gene. Clone pQWF contains the 3' half of the gene alone. The location of the PCR primers used to generate pQWF is indicated.

Figure 19 is a construction diagram for producing plasmid pQWE expressing a C-terminal portion of the 200 kDa protein of *M. catarrhalis* strain 4223 fused to the N-terminus;

Figure 20 is a construction diagram for producing plasmid pQWF expressing a C-terminal portion of the 200 kDa protein of *M. catarrhalis* strain 4223;

Figure 21 shows the nucleotide sequence (SEQ ID No: 5 - entire sequence, SEQ ID No: 6 - coding sequence) of the gene encoding the about 200 kDa outer membrane protein of *M. catarrhalis* strain 4223, as determined from PCR-amplified genomic DNA of strain 4223 and the deduced amino acid sequence (SEQ ID No: 7) of the corresponding about 200 kDa outer membrane protein and hence contains the same sequences as Figure 3. The Figure shows the identity of pKS348, containing a 5'-terminal truncation of the 200 kDa gene, the identity of pQWF (nucleotide sequence - SEQ ID No: 45, derived amino acid sequence, SEQ ID No: 46) comprising a 3' terminal half of the gene, such as those potential truncation sites and certain restrictions sites (see Figure 22);

Figure 22 shows the identity of certain restrictions sites (some of which are identified in Figure 21) is the full length gene encoding the about 200 kDa outer membrane protein of *M. catarrhalis* strain 4223 and the identity of truncations r200 kDa from pKS348, 3' r200 kDa from pQWF and 3' r200 kDa(t);

5        Figure 23 is a schematic procedure for producing plasmid pBR T7 3'rkDa(t)/KanR/cer from pQWF and 3' 200 kDa(t); and

Figure 24 contains SDS-PAGE analyses of cell lysates of transformed *E. coli* strains BL21-SI and BL21(DE3) expressing carboxy-terminated protein.

#### GENERAL DESCRIPTION OF THE INVENTION

10        In WO 96/34960 (Figure 6), the sequence of a cloned gene from *M. catarrhalis* 4223 encoding an about 200 kDa protein, was described. The open reading frame was predicted to start at a GTG codon. Sequence analysis of 200 kDa genes from additional strains, suggested that a slightly longer open reading frame was more generally found. A re-examination of the sequence from the lambda  
15        phage-derived 200 kDa gene confirmed the GTG start codon and an upstream stretch of 10 G nucleotides in a G tract. However, when sequence analysis was performed on 4223 genomic PCR-amplified subclones, the longer open reading frame was found starting from an ATG codon. The G-tract was found to contain 9 G nucleotides in the chromosomal gene. An additional G nucleotide had been inserted  
20        during cloning from the phage library. Analysis of the 5' end of the 200 kDa gene from 24 strains suggests that the number of G nucleotides in the G tract acts as regulator of expression.

Utilizing the techniques described herein, the genes encoding the about 200 kDa protein from *M. catarrhalis* strains Q8 and LES-1 have been cloned and  
25        sequenced. Figures 4 and 5 show respectively the nucleotide and derived amino acid sequences. An amino acid sequence comparison of the derived amino acid sequences of the 200 kDa protein from the three strains of *M. catarrhalis* is contained in Figure 6.

Based on the sequence information, a plasmid (pKS294) was constructed  
30        that contained the full-length 200 kDa protein gene of strain 4223 starting at the ATG codon, under control of the bacteriophage T7 promoter. However, even a basal level of expression of the full-length gene from the ATG was lethal to *E. coli*.

Deletion of a 165 bp 5' fragment of the 200 kDa coding region greatly reduced the toxicity of the resultant protein to *E. coli*. Plasmid pKS348 contains the T7 promoter transcriptionally driving a 200 kDa protein gene which starts at amino acid residue 56. The V56 codon was changed to M56. The M56 r200 kDa protein was produced  
5 and the purified protein was used to generate guinea pig antiserum.

In WO 96/34960, a bactericidal antibody assay was described that was used to demonstrate that anti-200 kDa antibody was bactericidal for *M. catarrhalis*. The assay was used herein to demonstrate broad bactericidal antibody activity against heterologous clinical isolates from different geographical locations, by anti-M56  
10 r200 kDa antibody. A single anti-M56 r200 kDa antibody was lytic for 62% of strains tested.

The 200 kDa protein was originally identified as a putative adhesin when its presence was detected in a clumping strain, but not a non-clumping derivative. In order to determine whether it were truly an adhesin, an *in vitro* adherence assay was  
15 developed in which the inhibition of binding by antibody between *M. catarrhalis* and epithelial cells was measured. Using this assay, anti-M56 r200 kDa antibody was capable of inhibiting adherence of the homologous strain by 48%, demonstrating that the 200 kDa protein was an adhesin. When an additional 25 strains of *M. catarrhalis* were assayed, 21 were found to have reduced adherence to  
20 epithelial cells in the presence of anti-M56 r200 kDa antibody. 19 of these strains had not been killed by the same antibody. Thus, a single anti-M56 r200 kDa antibody was capable of killing or blocking adherence of 91% of the strains tested.

The sequence comparison for the 200 kDa gene from three strains of *M. catarrhalis* showed that the C-terminal half of the protein was quite conserved.  
25 Strain LES-1 contained an insert of about 300 amino acids. Thus, based upon the C-terminal region, the strains may be divided into two families depending upon whether they contained the insert 4223 and Q8 formed one family while LES-1 formed the other. The carboxy terminal halves (3' halves) of the 4223 or LES-1 200 kDa genes were expressed in *E. coli* with good yields and the purified carboxy  
30 terminal half of the proteins were used to generate antibodies. When tested in the bactericidal antibody assay, these antisera were bactericidal, as seen in Table 1B.

It is clearly apparent to one skilled in the art, that the various embodiments of the present invention have many applications in the fields of vaccination, diagnosis, treatment of *Moraxella* infections, and in the generation of immunological reagents. A further non-limiting discussion of such uses is further presented below.

#### 1. Vaccine Preparation and Use

Immunogenic compositions, including those suitable to be used as vaccines, may be prepared from the about 200 kDa outer membrane protein as disclosed herein, as well as immunological fragments and fusions thereof, which may be purified from the bacteria or which may be produced recombinantly. The vaccine elicits an immune response in a subject which produces antibodies, including anti-200 kDa outer membrane protein antibodies and antibodies that are opsonizing or bactericidal. Should the vaccinated subject be challenged by *Moraxella* or other bacteria that produce proteins capable of producing antibodies that specifically recognize 200 kDa outer membrane protein, the antibodies bind to and inactivate the bacterium. Furthermore, opsonizing or bactericidal anti-200 kDa outer membrane protein antibodies may also provide protection by alternative mechanisms.

Immunogenic compositions including vaccines may be prepared as injectables, as liquid solutions or emulsions. The about 200 kDa outer membrane protein may be mixed with pharmaceutically acceptable excipients which are compatible with the about 200 kDa outer membrane protein. Such excipients may include, water, saline, dextrose, glycerol, ethanol, and combinations thereof. The immunogenic compositions and vaccines may further contain auxiliary substances, such as wetting or emulsifying agents, pH buffering agents, or adjuvants to enhance the effectiveness thereof. Immunogenic compositions and vaccines may be administered parenterally, by injection subcutaneously or intramuscularly. Alternatively, the immunogenic compositions formed according to the present invention, may be formulated and delivered in a manner to evoke an immune response at mucosal surfaces. Thus, the immunogenic composition may be administered to mucosal surfaces by, for example, the nasal or oral (intra-gastric) routes. Alternatively, other modes of administration including suppositories and oral formulations may be desirable. For suppositories, binders and carriers may

include, for example, polyalkylene glycols or triglycerides. Oral formulations may include normally employed excipients such as, for example, pharmaceutical grades of saccharine, cellulose and magnesium carbonate. These compositions can take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain about 1 to 95% of the about 200 kDa outer membrane protein. The immunogenic preparations and vaccines are administered in a manner compatible with the dosage formulation, and in such amount as will be therapeutically effective, protective and immunogenic. The quantity to be administered depends on the subject to be treated, including, for example, the capacity of the individual's immune system to synthesize antibodies, and if needed, to produce a cell-mediated immune response. Precise amounts of active ingredient required to be administered depend on the judgement of the practitioner. However, suitable dosage ranges are readily determinable by one skilled in the art and may be of the order of micrograms of the about 200 kDa outer membrane protein. Suitable regimes for initial administration and booster doses are also variable, but may include an initial administration followed by subsequent administrations. The dosage may also depend on the route of administration and will vary according to the size of the host.

The immunogenic preparations including vaccines may comprise as the immunostimulating material a nucleotide vector comprising at least a portion of the gene encoding the about 200 kDa protein, or the at least a portion of the gene may be used directly for immunization.

The concentration of the about 200 kDa outer membrane antigen in an immunogenic composition according to the invention is in general about 1 to 95%. A vaccine which contains antigenic material of only one pathogen is a monovalent vaccine. Vaccines which contain antigenic material of several pathogens are combined vaccines and also belong to the present invention. Such combined vaccines contain, for example, material from various pathogens or from various strains of the same pathogen, or from combinations of various pathogens.

Immunogenicity can be significantly improved if the antigens are co-administered with adjuvants, commonly used as 0.05 to 0.1 percent solution in phosphate-buffered saline. Adjuvants enhance the immunogenicity of an antigen

but are not necessarily immunogenic themselves. Adjuvants may act by retaining the antigen locally near the site of administration to produce a depot effect facilitating a slow, sustained release of antigen to cells of the immune system. Adjuvants can also attract cells of the immune system to an antigen depot and  
5 stimulate such cells to elicit immune responses.

Immunostimulatory agents or adjuvants have been used for many years to improve the host immune responses to, for example, vaccines. Intrinsic adjuvants, such as lipopolysaccharides, normally are the components of the killed or attenuated bacteria used as vaccines. Extrinsic adjuvants are immunomodulators which are  
10 typically non-covalently linked to antigens and are formulated to enhance the host immune responses. Thus, adjuvants have been identified that enhance the immune response to antigens delivered parenterally. Some of these adjuvants are toxic, however, and can cause undesirable side-effects, making them unsuitable for use in humans and many animals. Indeed, only aluminum hydroxide and aluminum  
15 phosphate (collectively commonly referred to as alum) are routinely used as adjuvants in human and veterinary vaccines. The efficacy of alum in increasing antibody responses to diphtheria and tetanus toxoids is well established and a HBsAg vaccine has been adjuvanted with alum. While the usefulness of alum is well established for some applications, it has limitations. For example, alum is  
20 ineffective for influenza vaccination and inconsistently elicits a cell mediated immune response.

A wide range of extrinsic adjuvants can provoke potent immune responses to antigens. These include saponins complexed to membrane protein antigens (immune stimulating complexes), pluronic polymers with mineral oil, killed  
25 mycobacteria in mineral oil, Freund's complete adjuvant, bacterial products, such as muramyl dipeptide (MDP) and lipopolysaccharide (LPS), as well as lipid A, and liposomes.

To efficiently induce humoral immune responses (HIR) and cell-mediated immunity (CMI), immunogens are typically emulsified in adjuvants. Many  
30 adjuvants are toxic, inducing granulomas, acute and chronic inflammations (Freund's complete adjuvant) FCA, cytotoxicity (saponins and Pluronic polymers) and pyrogenicity, arthritis and anterior uveitis (LPS and MDP). Although FCA is an

excellent adjuvant and widely used in research, it is not licensed for use in human or veterinary vaccines because of its toxicity.

Desirable characteristics of ideal adjuvants include:

- (1) lack of toxicity;
- 5 (2) ability to stimulate a long-lasting immune response;
- (3) simplicity of manufacture and stability in long-term storage;
- (4) ability to elicit both CMI and HIR to antigens administered by various routes, if required;
- (5) synergy with other adjuvants;
- 10 (6) capability of selectively interacting with populations of antigen presenting cells (APC);
- (7) ability to specifically elicit appropriate  $T_H1$  or  $T_H2$  cell-specific immune responses; and
- (8) ability to selectively increase appropriate antibody isotype levels (for  
15 example, IgA) against antigens.

U.S. Patent No. 4,855,283 granted to Lockhoff et al on August 8, 1989 which is incorporated herein by reference thereto, teaches glycolipid analogues including N-glycosylamides, N-glycosylureas and N-glycosylcarbamates, each of which is substituted in the sugar residue by an amino acid, as immuno-modulators or  
20 adjuvants. Thus, Lockhoff et al. (US Patent No. 4,855,283 and ref. 27) reported that N-glycolipid analogs displaying structural similarities to the naturally-occurring glycolipids, such as glycosphospholipids and glycoglycerolipids, are capable of eliciting strong immune responses in both herpes simplex virus vaccine and pseudorabies virus vaccine. Some glycolipids have been synthesized from long  
25 chain-alkylamines and fatty acids that are linked directly with the sugars through the anomeric carbon atom, to mimic the functions of the naturally occurring lipid residues.

U.S. Patent No. 4,258,029 granted to Moloney, assigned to the assignee hereof and incorporated herein by reference thereto, teaches that octadecyl tyrosine  
30 hydrochloride (OTH) functioned as an adjuvant when complexed with tetanus toxoid and formalin inactivated type I, II and III poliomyelitis virus vaccine. Also, Nixon-George et al. (ref. 24), reported that octadecyl esters of aromatic amino acids



complexed with a recombinant hepatitis B surface antigen, enhanced the host immune responses against hepatitis B virus.

Lipidation of synthetic peptides has also been used to increase their immunogenicity. Thus, Wiesmuller (ref. 25) describes a peptide with a sequence homologous to a foot-and-mouth disease viral protein coupled to an adjuvant tripalmityl-S-glyceryl-cysteinylserine, being a synthetic analogue of the N-terminal part of the lipoprotein from Gram negative bacteria. Furthermore, Deres et al. (ref. 26) reported *in vivo* priming of virus-specific cytotoxic T lymphocytes with synthetic lipopeptide vaccine which comprised of modified synthetic peptides derived from influenza virus nucleoprotein by linkage to a lipopeptide, N-palmityl-S-2,3-bis(palmitylxy)-(2RS)-propyl-[R]-cysteine (TPC).

## 2. Immunoassays

The about 200 kDa outer membrane protein of the present invention is useful as an immunogen for the generation of anti-200 kDa outer membrane protein antibodies, as an antigen in immunoassays including enzyme-linked immunosorbent assays (ELISA), RIAs and other non-enzyme linked antibody binding assays or procedures known in the art for the detection of anti-bacterial, anti-*Moraxella*, and anti-200 kDa outer membrane protein antibodies. In ELISA assays, the about 200 kDa outer membrane protein is immobilized onto a selected surface, for example, a surface capable of binding proteins such as the wells of a polystyrene microtiter plate. After washing to remove incompletely adsorbed about 200 kDa outer membrane protein, a nonspecific protein such as a solution of bovine serum albumin (BSA) that is known to be antigenically neutral with regard to the test sample may be bound to the selected surface. This allows for blocking of nonspecific adsorption sites on the immobilizing surface and thus reduces the background caused by nonspecific bindings of antisera onto the surface.

The immobilizing surface is then contacted with a sample, such as clinical or biological materials, to be tested in a manner conducive to immune complex (antigen/antibody) formation. This may include diluting the sample with diluents, such as solutions of BSA, bovine gamma globulin (BGG) and/or phosphate buffered saline (PBS)/Tween. The sample is then allowed to incubate for from 2 to 4 hours, at temperatures such as of the order of about 20° to 37°C. Following incubation, the

sample-contacted surface is washed to remove non-immunocomplexed material. The washing procedure may include washing with a solution, such as PBS/Tween or a borate buffer. Following formation of specific immunocomplexes between the test sample and the bound about 200 kDa outer membrane protein, and subsequent  
5 washing, the occurrence, and even amount, of immunocomplex formation may be determined by subjecting the immunocomplex to a second antibody having specificity for the first antibody. If the test sample is of human origin, the second antibody is an antibody having specificity for human immunoglobulins and in general IgG. To provide detecting means, the second antibody may have an  
10 associated activity such as an enzymatic activity that will generate, for example, a colour development upon incubating with an appropriate chromogenic substrate. Quantification may then be achieved by measuring the degree of colour generation using, for example, a visible spectrophotometer.

### 3. Use of Sequences as Hybridization Probes

15 The nucleotide sequences of the present invention, comprising the sequence of the about 200 kDa protein gene, now allow for the identification and cloning of the about 200 kDa protein gene from any species of *Moraxella*.

The nucleotide sequences comprising the sequence of the about 200 kDa protein gene of the present invention are useful for their ability to selectively form  
20 duplex molecules with complementary stretches of other about 200 kDa protein genes. Depending on the application, a variety of hybridization conditions may be employed to achieve varying degrees of selectivity of the probe toward the other genes. For a high degree of selectivity, relatively stringent conditions are used to form the duplexes, such as low salt and/or high temperature conditions, such as  
25 provided by 0.02 M to 0.15 M NaCl at temperatures of between about 50°C to 70°C. For some applications, less stringent hybridization conditions are required such as 0.15 M to 0.9 M salt, at temperatures ranging from between about 20°C to 55°C. Hybridization conditions can also be rendered more stringent by the addition of increasing amounts of formamide, to destabilize the hybrid duplex. Thus, particular  
30 hybridization conditions can be readily manipulated, and will generally be a method of choice depending on the desired results. In general, convenient hybridization temperatures in the presence of 50% formamide are: 42°C for a probe which is 95 to

100% homologous to the target fragment, 37°C for 90 to 95% homology and 32°C for 85 to 90% homology.

In a clinical diagnostic embodiment, the nucleic acid sequences of the about 200 kDa protein genes of the present invention may be used in combination with an appropriate means, such as a label, for determining hybridization. A wide variety of appropriate indicator means are known in the art, including radioactive, enzymatic or other ligands, such as avidin/biotin and digoxigenin-labelling, which are capable of providing a detectable signal. In some diagnostic embodiments, an enzyme tag such as urease, alkaline phosphatase or peroxidase, instead of a radioactive tag may be used. In the case of enzyme tags, colorimetric indicator substrates are known which can be employed to provide a means visible to the human eye or spectrophotometrically, to identify specific hybridization with samples containing about 200 kDa protein gene sequences.

The nucleic acid sequences of the about 200 kDa protein genes of the present invention are useful as hybridization probes in solution hybridizations and in embodiments employing solid-phase procedures. In embodiments involving solid-phase procedures, the test DNA (or RNA) from samples, such as clinical samples, including exudates, body fluids (e. g., serum, amniotic fluid, middle ear effusion, sputum, bronchoalveolar lavage fluid) or even tissues, is adsorbed or otherwise affixed to a selected matrix or surface. The fixed, single-stranded nucleic acid is then subjected to specific hybridization with selected probes comprising the nucleic acid sequences of the about 200 kDa protein encoding genes or fragments or analogs thereof of the present invention under desired conditions. The selected conditions will depend on the particular circumstances based on the particular criteria required depending on, for example, the G+C contents, type of target nucleic acid, source of nucleic acid, size of hybridization probe etc. Following washing of the hybridization surface so as to remove non-specifically bound probe molecules, specific hybridization is detected, or even quantified, by means of the label. It is preferred to select nucleic acid sequence portions which are conserved among species of *Moraxella*. The selected probe may be at least 18bp and may be in the range of about 30 to 90 bp.

#### 4. Expression of the about 200 kDa Protein Gene

Plasmid vectors containing replicon and control sequences which are derived from species compatible with the host cell may be used for the expression of the genes encoding the about 200 kDa protein in expression systems. The vector  
5 ordinarily carries a replication site, as well as marking sequences which are capable of providing phenotypic selection in transformed cells. For example, *E. coli* may be transformed using pBR322 which contains genes for ampicillin and tetracycline resistance and thus provides an easy means for identifying transformed cells. The plasmids or phage, must also contain, or be modified to contain, promoters which  
10 can be used by the host cell for expression of its own proteins.

In addition, phage vectors containing replicon and control sequences that are compatible with the host can be used as a transforming vector in connection with these hosts. For example, the phage in lambda GEM™-11 may be utilized in making recombinant phage vectors which can be used to transform host cells, such  
15 as *E. coli* LE392.

Promoters commonly used in recombinant DNA construction include the -lactamase (penicillinase) and lactose promoter systems and other microbial promoters, such as the T7 promoter system as described in U.S. Patent No. 4,952,496. Details concerning the nucleotide sequences of promoters are known,  
20 enabling a skilled worker to ligate them functionally with genes. The particular promoter used will generally be a matter of choice depending upon the desired results. Hosts that are appropriate for expression of the about 200 kDa protein genes, fragments, analogs or variants thereof, may include *E. coli*, *Bacillus* species, *Haemophilus*, fungi, yeast, *Bordetella*, or the baculovirus expression system may be  
25 used.

In accordance with this invention, it is preferred to make the protein by recombinant methods, particularly when the naturally occurring about 200 kDa protein as purified from a culture of a species of *Moraxella* may include trace amounts of toxic materials or other contaminants. This problem can be avoided by  
30 using recombinantly produced protein in heterologous systems which can be isolated from the host in a manner to minimize contaminants in the purified material. Particularly desirable hosts for expression in this regard include Gram positive

bacteria which do not have LPS and are, therefore, endotoxin free. Such hosts include species of *Bacillus* and may be particularly useful for the production of non-pyrogenic about 200 kDa protein, fragments or analogs thereof.

#### BIOLOGICAL DEPOSITS

5 Certain plasmids that contain portions and full-length of the gene having the open reading frame of the gene encoding the about 200 kDa outer membrane protein of *M. catarrhalis* strain 4223 that are described and referred to herein have been deposited with the America Type Culture Collection (ATCC) located at 10801 University Blvd., Manassas, VA 20110-2209, U.S.A., pursuant to the Budapest  
10 Treaty and pursuant to 37 CFR 1.808 and prior to the filing of this application.

Samples of the deposited plasmids will become available to the public upon grant of a patent based upon this United States patent application or relevant precursor applications. The invention described and claimed herein is not to be limited in scope by plasmids deposited, since the deposited embodiment is intended  
15 only as an illustration of the invention. Any equivalent or similar plasmids that encode similar or equivalent antigens as described in this application are within the scope of the invention.

	<u>Plasmid</u>	<u>ATCC Designation</u>	<u>Date Deposited</u>
	pKS47	97,111	April 7, 1995
20	pKS5	97,110	April 7, 1995
	pKS9	97,114	April 18, 1995
	pKS294	203,528	December 17, 1998
	pKS348	203,529	December 17, 1998

#### EXAMPLES

25 The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific Examples. These Examples are described solely for purposes of illustration and are not intended to limit the scope of the invention. Changes in form and substitution of equivalents are contemplated as circumstances may suggest or render expedient.  
30 Although specific terms have been employed herein, such terms are intended in a descriptive sense and not for purposes of limitations.

Methods of molecular genetics, protein biochemistry, and immunology used but not explicitly described in this disclosure and these Examples are amply reported in the scientific literature and are well within the ability of those skilled in the art.

#### Example 1

5        This Example describes the cloning of a gene encoding the *M. catarrhalis* 200 kDa outer membrane protein.

         A *M. catarrhalis* genomic library in phage lambda EMBL3 was prepared as described in Example 9 of USP 5,808,024 and WO 96/34960 and was screened using guinea pig anti-200 kDa protein antiserum. A lambda phage clone 8II, which  
10       expressed an about 200 kDa protein, was confirmed by immunoblotting of the phage lysate using the about 200 kDa outer membrane-specific antiserum.

         Plate lysate cultures of this recombinant phage were prepared. The DNA was extracted from the plate lysates using a Wizard Lambda Preps DNA Purification System (Promega Corp, Madison, WI) according to the manufacturer's instructions.  
15       This phage clone carried a DNA insert of about 16 kb in size (the restriction map for which is shown in Figure 1). The phage DNA was digested with a mixture of the restriction enzymes *SalI* and *XhoI*, and separated by agarose gel electrophoresis. Two DNA bands, approximately 5 kb and 11 kb in size, respectively, were cut out from the gel and extracted using a Geneclean kit (BIO 101 Inc., LaJolla, CA)  
20       according to the manufacturer's direction.

         The smaller 5 kb fragment was ligated into a plasmid vector, pBluescript II SK +/- (Stratagene Cloning Systems, LaJolla, CA), which had been previously digested with *SalI* and *XhoI*, to produce plasmid pKS5. The larger 11 kb fragment was ligated into a plasmid vector, pSP72 (Promega Corp., Madison, WI), digested  
25       with *SalI* and *XhoI*, to produce plasmid pKS9. Both ligated plasmids were used to transform *E. coli*, strain DH5.

         The lambda phage DNA was also digested with a mixture of *XhoI* and *KpnI* and the approximately 1.1 kb fragment was isolated after agarose gel separation as described above. This 1.1 kb fragment was ligated into a plasmid vector, pGEM-  
30       7Zf(+) (Promega Corp., Madison, WI), to produce plasmid pKS47.

### Example 2

This Example describes the isolation of chromosomal DNA from *M. catarrhalis* for use in PCR amplification.

*M. catarrhalis* was cultured in 25 ml of BHI broth overnight and centrifuged  
5 at 5,000 rpm for 10 min. The bacteria pellet was suspended in 10 ml of 10 mM Tris/HCl (pH 8.0) containing 100 mM EDTA and mixed with RNaseA (final concentration: 100 µg/ml) and lysozyme (final concentration: 1 mg/ml). After incubation on ice for 10 min and at room temperature for 50 min, the suspension was gently mixed with 1 ml of 10% SDS and then heated at 65°C for 20 min. The  
10 suspension was mixed with proteinase K (final concentration: 200 µg/ml) and incubated at 50°C for 1 h. The suspension was gently mixed with 10 ml chloroform on a nutator for 15 min and centrifuged at 5,000 rpm for 10 min. The upper phase was slowly removed with a wide-bore pipette and mixed with 10 ml of Tris-saturated phenol and 10 ml of chloroform on a nutator. After centrifugation at 5,000  
15 rpm for 10 min, the upper phase was re-extracted with a mixture of Tris-saturated phenol and chloroform, again, and then extracted with chloroform, and then twice dialyzed against 1M NaCl at 4°C and twice against TE buffer (pH 8.0) at 4°C.

### Example 3

This Example describes subcloning and sequence analysis of fragments of  
20 the 200 kDa protein gene from *M. catarrhalis* strain 4223.

The procedures used to produce a phage λEMBL3 clone 8II, and its subclones, pKS5, pKS9 and pKS47, are described in USP 5,808,024 and WO 96/34960. pKS10 was constructed from the λEMBL3 clone 8II exactly as described for pKS9. pKS59 and pKS63 were constructed by insertion of a 1.4 kb *XbaI-NcoI*  
25 fragment of pKS9 into pGEM5Z(+) that had been digested with *NcoI* and *SpeI*. pKS71 was made by insertion of the same 1.4 kb *XbaI-NcoI* fragment, isolated from the λEMBL3 clone 8II into pGEM5Z(+). Sequence analysis confirmed that all three plasmids, pKS59, pKS63 and pKS71, carried identical DNA fragments. Figure 1 shows partial restriction maps for the plasmids.

30 The full sequence of the 200 kDa gene locus from the λDNA clone was described in USP 5,808,024 and WO 96/34960 and is shown in Figure 2. There is a tract of 10 consecutive G nucleotides between position 623 and 632 in clones

derived from the  $\lambda$  library. The first possible start codon is, therefore, located at nucleotides 706 to 708 and is a GTG encoding a valine, boxed lightly in Figure 2. A series of strains expressing a 200 kDa gene, were identified by immunoblot analysis and the 5' end of their 200 kDa genes was PCR amplified and sequenced. A summary of the findings is shown in Table 5 wherein the expression level of the gene appeared to be related to the number of G nucleotides in the tract and for those strains within higher expression levels, the start codon was an ATG upstream of the GTG codon identified from the 4223  $\lambda$  clones. Based upon these findings, the sequence of the 5' end of the 200 kDa gene from strain 4223 was re-examined.

Plasmids pKS9 and pKS10 were directly derived from the  $\lambda$  clone. The subclones pKS59 and pKS63 were derived from pKS9 whereas pKS71 contained the same fragment derived directly from the  $\lambda$  clone. All of these plasmids contained 10 G nucleotides in the G tract, as described previously. To determine whether the  $\lambda$  clone contained an extra G nucleotide or the strain itself contained an aberrant gene, PCR amplification of the region was performed from chromosomal DNA preparations and from the  $\lambda$  subclones. The data in Table 3 show that PCR fragments of the  $\lambda$  subclones all contained 10 G nucleotides. The data in Table 4, however, demonstrate that PCR fragments derived directly from chromosomal DNA, contain 9 G nucleotides in the tract. When the single extra G nucleotide is removed from the 200 kDa sequence of strain 4223, the open reading frame is extended in the 5' direction to start from an ATG codon 156 nucleotides earlier, at positions 541 to 543 in Figure 2. This new start codon corresponds to that suggested for the 200 kDa genes sequenced from other strains and summarized in Table 5.

#### Example 4

This Example describes the construction of the full length 200 kDa protein gene from *M. catarrhalis* strain 4223. The construction scheme is shown in Figure 9.

The full-length 200 kDa protein gene was constructed from the new ATG start codon identified by analysis of the chromosomally derived DNA as described in Example 3 and shown in Figure 3. pKS47 was digested with *Xho*I and *Kpn*I and separated by agarose gel electrophoresis. The 1.1 kb fragment was isolated from the gel and inserted into pKS5, which had previously been digested with the same two



enzymes and purified to form pKS80. An about 5.8 kb *Pst*I fragment from pKS80 was inserted into pT7-7 vector (ref. 28) that had been digested with *Pst*I and dephosphorylated. The orientation of the insert was determined by restriction enzyme analysis and pKS122 was chosen for further construction (see Figure 7).

5           The 5' region of the 200 kDa protein gene was amplified from strain 4223 chromosomal DNA. PCR reactions were performed using Taq Plus or Tsg Plus enzyme (Sangon Ltd., Scarborough, Ont., Canada) and a Perkin Elmer DNA Thermocycler (Perkin Elmer Cetus, Foster City, CA, USA). The lower PCR reaction mixture (50 µl) contained 5 µl of 10X buffer, 0.4 mM each of four deoxynucleotide  
10       triphosphates (Perkin Elmer, Foster City, CA, USA) and 1 to 2 µM each of two primers. The upper PCR reaction mixture (50 µM) contained 5 µl of 10X buffer, 0.5 to 1 µl of Taq Plus or Tsg Plus enzyme, and template DNA. The lower and upper mixtures were separated by a layer of AmpliWax PCR Gem50 (Perkin Elmer, Foster City, CA, USA) before heating cycles started. The thermocycling condition  
15       employed for the provision of PCR products in the construction of various plasmids are set forth in Table 11 below. The PCR products were purified using a QIAquick PCR purification kit (Qiagen Inc., Mississauga, Ont., Canada). The purified PCR products were sequenced on both strands directly and/or after cloning in appropriate vectors using an Applied Biosystem sequencer.

20           The 5' primer (designated 5295.KS) was designed, so that it contained the first possible translation start codon, ATG, and its flanking sequences with a mutation to introduce an *Nde*I site at the ATG. The 3' primer (designated 4260.KS) was based upon the non-coding strand in the region about 1 kb downstream from the ATG start codon. (The nucleic acid sequences and SEQ ID's of the PCR primers  
25       utilized herein are identified in Table 10). The PCR-product was digested with *Nde*I and an approximately 650 bp DNA fragment was gel purified and inserted into pKS122, which had previously been linearized with *Nde*I and dephosphorylated.

          The new construct, designated pKS294 (Figure 8), was confirmed by restriction enzyme analyses and by sequencing of the PCR-amplified DNA and its  
30       joint regions. The number of G nucleotides in the G tract was nine, and the open reading frame continued from the newly found translation start codon, ATG, to the remaining portion of 200 kDa protein gene in pKS122. pKS294, therefore, carried

the correct, full-length 200 kDa protein gene from *Moraxella catarrhalis* strain 4223. During construction of pKS294, *E. coli* strain DH5 $\alpha$  was used for transformation and plasmid analyses.

#### Example 5

5        This Example describes the cloning and sequence analysis of genes encoding the 200 kDa protein from additional *M. catarrhalis* clinical isolates.

         A panel of *M. catarrhalis* clinical isolates was analysed by immunoblot with guinea pig anti-200 kDa antibody, as described in USP 5,808,024 and WO 96/34960. From these analyses, it was evident that there is size heterogeneity among  
10    the 200 kDa proteins from various strains. In order to assess the possible genetic heterogeneity, representative strains were chosen for gene cloning. Strain Q8 is a naturally occurring relatively non-clumping strain that produces a 200 kDa protein of about the same size as the 4223-derived protein. Strain LES-1 produces a larger 200 kDa protein. These strains were also selected based upon bactericidal antibody  
15    data as illustrated in Table 1. The 200 kDa genes were cloned from these two strains of *M. catarrhalis* and sequenced.

         The nucleotide and derived amino acid sequences of the 200 kDa genes from strains Q8 and LES-1 are shown in Figures 4 and 5 respectively. An alignment of the amino acid sequences with the 4223-derived sequence is shown in Figure 6. As  
20    can be seen, the first 68 residues of the N-terminus are quite conserved, especially between strains 4223 and Q8. In addition, the final 456 residues of the C-terminus are nearly identical among the three strains. The remainder of the sequence has regions of high homology and significant diversity, including an insert of more than 300 residues for strain LES-1.

25        The N-terminal sequence of the 200 kDa proteins is homologous to the *H. influenzae* Hia and Hsf proteins, as well as other high molecular weight proteins or adhesins, such as AIDA (ref. 33).

         The C-terminal region also has some homology to *H. influenzae* Hia and Hsf proteins as do some stretches of internal sequence. There is also some homology in  
30    the C-terminal region to UspA (ref. 23). A further indication of the relatedness of this family of proteins, is the finding that guinea pig anti-200 kDa antibody raised to gel-purified native protein was able to recognize recombinant Hia protein by

immunoblot. This data has been described in copending United States Patent Application No. 09/268,347 filed March 16, 1999, assigned to the assignee hereof and the disclosure of which is incorporated herein by reference.

#### Example 6

5           This Example shows the expression of the full-length about 200 kDa protein from pKS294.

*E. coli* strain, BL21(DE3)/pLysS was transformed by electroporation with pKS294, prepared as described in Example 4, for the expression study of the full-length 200 kDa protein gene.

10           The product of the pKS294 construct was found to be toxic to the host *E. coli*. At room temperature, the BL21(DE3)/pLysS transformants grew very slowly on LB-agar plates containing ampicillin (Amp) and chloramphenicol (Cm) and at 37°C, no transformants were detected. When the transformants which grew at room temperature, were cultured overnight at 30°C on BHI agar containing the two  
15   antibiotics and glucose, they grew well, producing colonies with a normal size. However, when these clones were cultured overnight in liquid medium at 30°C, subcultured into broth without glucose, and then induced by addition of IPTG, no recombinant protein was found on Western blot using anti-200 kDa protein serum. When the cells cultured overnight were examined before subculturing, a small  
20   quantity of recombinant 200 kDa protein was detected by SDS-PAGE stained with Coomassie Blue and by Western blot, showing that the gene was expressed during the overnight culture.

          When *E. coli* strain, DH5 $\alpha$ , which cannot express the gene under the control of a T7 promoter, was transformed with pKS294, the transformants grew well at  
25   37°C both on LB-agar and in LB-broth containing the antibiotics. These results suggest that the gene product is very toxic to host *E. coli*, and that even a basal level of expression of the full-length 200 kDa protein gene from the ATG is lethal to *E. coli*.

*M. catarrhalis* strain LES-1 also produced similar toxicity in *E. coli* when  
30   the full length 200 kDa protein was expressed.

### Example 7

This Example describes the deletion of a short 5'-sequence from the strain 4223 or strain LES-1 200 kDa protein gene and expression of the truncated genes producing a M56 r200 kDa product.

5        The deletion of a short 5' region from the strain 4223 200 kDa protein gene is shown in Figure 10 and was performed using a similar approach as described in Example 4. An about 500 bp 5' region of the 200 kDa gene was PCR amplified from strain 4223 using primers 5471.KS and 4257.KS (Table 8) from chromosomal DNA. The 5' primer (designated 5471.KS) was based upon the region surrounding  
10       the previously identified GTG downstream start codon. In primer 5471.KS, the flanking regions around the GTG codon were incorporated and the GTG was mutated to ATG with further mutations used to introduce an *NdeI* site incorporating the new ATG. Using numbering from the full-length 200 kDa protein, the new start codon would be M56 replacing the previous V56 codon. The 3' primer (designated  
15       4257.KS) was based upon the non-coding strand located about 500 bp downstream from the GTG codon in the 200 kDa protein gene. The PCR-product was digested with *NdeI*, purified using a QIAquick PCR purification kit (Qiagen Inc., Mississauga, Ont.), and inserted into *NdeI* digested and dephosphorylated pKS122 to provide pKS348 (see Figure 7). Plasmid pKS348 was confirmed by restriction  
20       enzyme analyses and by sequencing of the PCR-amplified DNA piece and its joint regions. The nucleotide sequence (SEQ ID No: 12) and the deduced amino acid sequence (SEQ ID No: 13) for the 5'-truncation contained in pKS348 are shown in Figure 8. A similar N-terminal truncated 200 kDa gene from strain LES-1 was generated in the same manner and was designated pKS444.

25       A single colony of *E. coli*, BL21(DE3)/pLysS, (KS358) which carried pKS348, was suspended in 5 ml of BHI broth containing Amp (100  $\mu$ M), Cm (50  $\mu$ M) and 0.4% of glucose, and cultured overnight at 37°C. To study the kinetics of expression, 2.5 ml of the overnight culture was added to 250 ml of LB (Luria-Bertani) broth containing Amp (100  $\mu$ M) and Cm (50  $\mu$ M), and grown with shaking  
30       at 37°C to  $A_{600} = 0.33$  to 0.36. Another 0.3 ml of the overnight culture was added to 30 mL of LB broth containing Amp (100  $\mu$ M) and Cm (50  $\mu$ M) and grown with shaking at 37°C to  $A_{600} = 0.26$  to 0.44. Gene expression from the cultures was

induced by addition of IPTG (final concentration: 4 mM). The bacteria were grown and harvested at different time points by centrifugation. The expression of the 200 kDa protein gene in the culture was confirmed by SDS-PAGE analysis using Coomassie Blue staining and by Western blot analysis using guinea pig anti-200 kDa protein serum, as described in USP 5,808,024 and WO 96/34960.

When *E. coli* BL21(DE3)/pLysS was transformed with pKS348, transformants grew well even on LB agar plates and in LB broth containing antibiotics at 37°C. After induction with IPTG, these clones produced a large amount of the N-terminally truncated r200 kDa protein which was clearly seen by SDS-PAGE Coomassie Blue stain, as shown in Figure 12.

The bacterial culture induced at  $A_{600} = 0.26$  produced slightly more truncated r200 kDa protein than the culture induced when the OD reading was 0.44. The largest amount of truncated r200 kDa protein was seen at 3 hr after induction. Similar results were observed for the M56 r200 kDa expression from strain LES-1.

#### 15 Example 8

This Example describes the purification of the M56 r200 kDa proteins from strain 4223 or LES-1, according to the procedure shown in Figure 11.

*E. coli* cell pellets were obtained from 500 ml culture prepared as described in Example 7, by centrifugation and were resuspended in 50 ml of 50 mM Tris-HCl, pH 8.0, containing 0.1 M NaCl, and disrupted by sonication. The sonicate was centrifuged at 20,000 xg for 30 min. and the resultant supernatant (sup1) was discarded. The pellet (ppt1) was extracted, in 50 ml of 50 mM Tris-HCl, pH 8.0 containing 0.5% Triton X-100 and 10 mM EDTA, then centrifuged at 20,000 xg for 30 min. and the supernatant (sup2) was discarded. The pellet (ppt2) was further extracted in 50 ml of 50 mM Tris-HCl, pH 8.0, containing 1% octylglucoside, then centrifuged at 20,000 xg for 30 min. and the supernatant (sup3) was discarded.

The resultant pellet (ppt3) contained the inclusion bodies. The pellet was solubilized in 6 ml of 50 mM Tris-HCl, pH 8.0, containing 6 M guanidine and 5 mM DTT. Twelve ml of 50 mM Tris-HCl, pH 8.0 was added, the mixture centrifuged at 20,000 xg for 30 min, and the pellet (ppt4) discarded. The supernatant (sup4) was precipitated by adding polyethylene glycol (PEG) 4000 at a final concentration of 5% and incubated at 4°C for 30 min. The resultant pellet (ppt5) was

removed by centrifugation at 20,000 xg for 30 min. The supernatant was then precipitated by  $(\text{NH}_4)_2\text{SO}_4$  at 50% saturation at 4°C overnight. After the addition of  $(\text{NH}_4)_2\text{SO}_4$ , the solution underwent phase separation with protein going to the upper phase (as judged by the cloudiness of the layer). The upper phase was collected, then  
5 subjected to centrifugation at 20,000 xg for 30 min. The resultant pellet was collected and dissolved in 2 ml of 50 mM Tris-HCl, pH 8.0, containing 6 M guanidine and 5 mM DTT. The clear solution was purified on a Superdex 200 gel filtration column equilibrated in 50 mM Tris-HCl, pH 8.0, containing 2 M guanidine HCl. The fractions were analysed by SDS-PAGE and those containing  
10 the purified r200 kDa were pooled. The pooled fraction was concentrated 5 to 10 fold using a centrprep 30 and then dialysed overnight at 4°C against PBS, and centrifuged at 20,000 xg for 30 min to clarify.

The protein remained soluble under these conditions and glycerol was added to the M56 r200 kDa preparation at a final concentration of 20% for storage at -20°C  
15 (Figure 12). The average yield of the purified M56 r200 kDa protein is about 10 mg  $\text{L}^{-1}$  culture. The purified protein was used for the immunization of animals, as described below.

The procedure of this Example 8 and was repeated for *M. catarrhalis* strain LES-1 and a corresponding r200 kDa protein was produced. The N-terminal  
20 truncated M56 r200 kDa protein from strain LES-1 gave approximately the same recovery of purified protein as described above for strain 4223.

#### Example 9

This Example illustrates the immunogenicity of the M56 r200 kDa protein.

The immunogenicity of M56 r200 kDa, prepared as described in Example 8,  
25 was examined using mice and guinea pigs. Groups of five BALB/c mice (Charles River, Quebec) were immunized sub-cutaneously (s.c.) on days 1, 29 and 43 with 0.3, 1.3 and 10  $\mu\text{g}$  of 4223 M56 r200 kDa antigen, prepared as described in Example 8, in the presence of  $\text{AlPO}_4$  (1.5 mg per dose). Blood samples were collected on days 0, 14, 28, 42 and 56.

30 Groups of five guinea pigs (Charles River, Quebec) were immunized i.m. on days 1, 29 and 43 with 25, 50 and 100  $\mu\text{g}$  of 4223 M56 r200 kDa antigen prepared

as described in Example 8, in the presence of  $\text{AlPO}_4$  (1.5 mg per dose). Blood samples were collected on days 0, 14, 28, 42 and 56.

Anti-M56 r200 kDa IgG titers were determined by antigen-specific enzyme-linked immunosorbent assays (EIAs). Microtiter wells (Nunc-MAXISORP, Nunc, Denmark) were coated with 50  $\mu\text{L}$  of protein antigen ( $0.2 \mu\text{g mL}^{-1}$ ). The reagents used in the assays were as follows: affinity-purified F(ab')<sub>2</sub> fragments of goat anti-mouse IgG (Fc-specific) conjugated to horseradish peroxidase (Jackson ImmunoResearch Labs, Mississauga, Ontario); affinity-purified guinea pig anti-IgG antibody ( $1 \mu\text{g mL}^{-1}$ )(prepared by the inventors); and affinity-purified F(ab')<sub>2</sub> fragment of goat anti-guinea pig IgG (H+L) antibodies conjugated to horseradish peroxidase (HRP) (Jackson ImmunoResearch Laboratories) used as a reporter. The reactions were developed using tetramethylbenzidine (TMB/ $\text{H}_2\text{O}_2$ , ADI, Mississauga, Ontario) and absorbancies were measured at 450 nm (using 540 nm as a reference wavelength) in a Flow Multiskan MCC microplate reader (ICN Biomedicals, Mississauga, Ontario). The reactive titer of an antiserum was defined as the reciprocal of the dilution consistently showing a two-fold increase in absorbance over that obtained with the pre-bleed serum sample.

The mice generated dose-dependent anti-M56 r200 kDa antibody responses, as shown in Figure 14. These results clearly show that the protein remained immunogenic after inclusion bodies extraction, solubilization and purification. Only a slight difference in the antibody titers were found for the higher dose range tested in guinea pigs (Figure 15), indicating that the amount of antigen used was nearly at saturation.

#### Example 10

This Example describes the generation of hyper-immune sera against the M56 r200 kDa proteins in rabbits and guinea pigs.

To generate hyper-immune sera against M56 r200 kDa proteins, groups of two rabbits and two guinea pigs (Charles River, Quebec) were immunized intramuscularly (i.m.) on day 1 with a 5  $\mu\text{g}$  dose of purified M56 r200 kDa protein, prepared as described in Example 8, emulsified in complete Freund's adjuvant (CFA). Animals were boosted on days 14 and 29 with the same dose of protein emulsified in incomplete Freund's adjuvant (IFA). Blood samples were taken on day

42 for analyzing the anti-M56 r200 kDa antibody titers and bactericidal activities. Anti-r200 kDa IgG titers were determined by antigen-specific enzyme-linked immunosorbent assays (EIAs), as described in Example 9. The results obtained in the two animals using r200 kDa protein from strains 4223 and LES-1 are illustrated in Table 6.

#### Example 11

This Example describes a bactericidal antibody assay.

The bactericidal antibody activity of guinea pig anti-M56 r200 kDa sera from 4223 or LES-1 protein prepared as described in Example 10 against various strains of *M. catarrhalis* was estimated using a viability plating assay. Each test strain of *M. catarrhalis* was cultured overnight in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, MI) at 37°C. The overnight culture was subcultured into 10 ml BHI broth, and grown to an absorbance at 578 nm of 0.5. The number of bacteria at  $A_{578} = 0.5$  changes from strain to strain. Therefore, several ten-fold dilutions of each strain were used in order to achieve 100 to 300 colonies per plate for the preimmune serum group. Bacteria were diluted in Veronal buffered saline (VBS, pH 7.6) containing 140 mM NaCl, 93 mM NaHCO<sub>3</sub>, 2 mM Na-barbiturate, 4 mM barbituric acid, 0.5 mM MgCl<sub>2</sub>·6H<sub>2</sub>O, 0.4 mM CaCl<sub>2</sub>·2H<sub>2</sub>O, and 0.1% bovine serum albumin. Guinea pig anti-M56 r200 kDa serum and pre-immune control serum were heated at 56°C for 30 min. to inactivate endogenous complement. Serum and antiserum were diluted in VBS, and placed on ice.

Twenty-five µl of diluted pre-immune serum or test antiserum were added to the wells of a 96-well Nunclon microtitre plate (Nunc, Roskilde, Denmark). Twenty-five µl of diluted bacterial cells were added to each of the wells. A guinea pig complement (BioWhittaker, Walkerville, MD) was diluted 1:10 in VBS, and 25 µl portions were added to each well. The plates were incubated for 60 min, gently shaking at 70 rpm on a rotary platform. Fifty µl of each reaction mixture were plated onto Mueller Hinton agar plates (Becton-Dickinson, Cockeysville, MD). The plates were incubated at 37°C for 24 hours, and then left at room temperature for a further 24 hours. The number of colonies per plate was counted, and average values of colonies per plate were estimated from duplicate pairs.



When pre-immune serum plates were compared with PBS control plates (no serum), pre-immune serum had no bactericidal effect on the homologous strain 4223. Therefore, it was assumed that the number of colonies per plate on pre-immune serum plates represented 100% viability for each strain and percent bactericidal killing was calculated as follows:

$$100\% - \left[ \frac{\text{average number of colonies per plate in anti-r200 kDa antiserum group} \times 100\%}{\text{average number of colonies per plate in pre-immune serum group}} \right]$$

When the bactericidal antibody activity of the 4223 anti-M56 r200 kDa antiserum was examined against the homologous strain (Table 7), 50% killing was observed at a serum dilution between 1/512 and 1/1024, showing that the antiserum raised against M56 r200 kDa protein possesses bactericidal antibody activity. Next, the bactericidal antibody activity of the antiserum was tested at a dilution of 1/64 against a total of 55 different strains, which were isolated from otitis media patients in various geographical locations (Table 1B). The antiserum raised against the M56 r200 kDa protein from strain 4223 showed more than 30% bactericidal antibody activity against 38 out of 56 (68%) strains examined. When LES-1 anti-M56 r200 kDa antibody was tested in the bactericidal antibody assay, 36/55 (65%) strains were killed, including 11 strains that were not killed by the 4223 anti-M56 r200 kDa antibody. Only six strains out of 55 strains examined were not killed by either one of the two antisera. These results indicate that the 200 kDa protein is a very good candidate for inclusion in an otitis media vaccine.

#### Example 12

This Example describes the inhibition of binding of *M. catarrhalis* strains to either Chang or Hep-2 epithelial cells by 4223 anti-M56 r200 kDa serum.

The 200 kDa protein had previously been proposed to be an adhesin on the basis of its apparent absence from a spontaneous non-clumping variant of strain 4223. This strain, obtained by serial passaging of culture supernatants, was designated RH408 and is described in WO 96/34960. Electron microscopy also suggested that the 200 kDa protein was an adhesin. The sequence homology demonstrated between the *M. catarrhalis* 200 kDa proteins and other high molecular weight adhesins from different organisms, also suggested that it was an adhesin. Based upon these observations, an assay was developed to try to demonstrate that

anti-r200 kDa antibody could block adherence between *M. catarrhalis* and epithelial cells, thus identifying it definitively as an adhesin.

On day 1, 24 well tissue culture plates were seeded with approximately  $3 \times 10^5$  Chang cells per well, to achieve a confluent monolayer following overnight  
5 incubation at 37°C in the presence of 5% CO<sub>2</sub>. *M. catarrhalis* 4223 or Q8 was cultured in 10 ml of BHI broth at 37°C for 18 hr, shaking at 200 rpm.

On day 2, bacterial cultures were pelleted by centrifugation at 3500 rpm for 10 min, and washed with 10 ml of PBS. After a centrifugation as above, each pellet was resuspended in 2 ml of DMEM supplemented with 10% FBS and 2 mM  
10 glutamine. The bacteria cultures were diluted 1/10 in the supplemented DMEM to OD of approximately 1.8 at 578 nm. Confluent monolayers of Chang cells were washed once with 1 ml of PBS per well, and 0.5 ml of 10% BSA in PBS was added to each well as a blocking agent. Plates were incubated at 37°C for 30 min and monolayers were washed twice with PBS as above.

15 A guinea pig anti-4223 M56 r200 kDa antiserum, prepared as described in Example 10 and pooled pre-immune guinea pig sera were heated at 56°C for 30 min to inactivate endogenous complement. Equal volumes of appropriately diluted antisera and bacteria were mixed, and 200 µl of the mixture were added into each well. Examples of antiserum dilutions tested included 1/4, 1/16 and 1/64. The plate  
20 was incubated at 37°C for 1 hr, with gentle shaking. The plate was carefully washed four times with 1 ml of PBS per well to remove the bacteria. To each well, 100 µl of trypsin were added, and the plate was incubated at 37°C for 5 min. After inactivation of trypsin by addition of 900 µl Dulbecco's Minimal Essential Medium (DMEM) to each well, the cells were resuspended by pipetting up and down several times.

25 Ten-fold dilutions of resuspended cells were prepared in a new 96-well plate. Fifty µl each of the  $1 \times 10^{-2}$ ,  $1 \times 10^{-3}$ ,  $1 \times 10^{-4}$  and  $1 \times 10^{-5}$  diluted samples were plated on a Mueller-Hinton agar plate. Plates were incubated at 37°C overnight, and then left at room temperature for a further 24 hours. The number of colonies per plate was counted for the estimation of the total bound bacteria.

30 Dilution plating was also carried out for each bacterial strain, to estimate bacterial concentrations and to calculate the total amount of bacteria added to each well. It was assumed that the number of bacteria bound to tissue culture cells in the

presence of pre-immune sera represented 100% optimal binding for each assay, and 0% inhibition. Therefore, in order to calculate the percent inhibition of the antiserum, we used the following formula:

$$\% \text{ inhibition} = 100 - \left[ \frac{\text{total bacteria bound in 4223 anti-r200 kDa antiserum samples} \times 100}{\text{total bacteria bound in pre-immune sera samples}} \right]$$

When the guinea pig 4223 anti-M56 r200 kDa protein serum was examined  
5 for the inhibition of binding of strain 4223 to Chang cells (Table 8), inhibition of 98%, 92% and 83% was observed at antiserum dilutions of 1/4, 1/16 and 1/64, respectively. With the heterologous strain Q8, the inhibition of binding to the tissue culture cells was estimated to be 77%, 82% and 55% at antiserum dilutions of 1/4, 1/16 and 1/64, respectively. The results clearly showed that anti-M56 r200 kDa  
10 protein serum inhibited the binding of *M. catarrhalis* to cultured human epithelial cells.

Having demonstrated that 4223 anti-M56 r200 kDa antibody could block adherence of *M. catarrhalis* strains 4223 or Q8 to Chang epithelial cells in a dose-dependent manner, the studies were extended to other strains. Of particular interest,  
15 were those strains that were not killed by anti-M56 r200 kDa antisera in the bactericidal antibody assay. To perform the *in vitro* adherence assay on several strains, a single antibody dilution of 1/16 was used. The data for inhibition of *in vitro* adherence to Hep-2 cells is summarized in Table 9. The procedure for the Hep-2 epithelial cells was identical to the Chang cell procedure described above. The  
20 4223 anti-M56 r200 kDa antibody effectively blocked adherence of the homologous strain by 48%. Strain RH408 does not express the 200 kDa gene and in the assay, antibody inhibited adherence of RH408 to 9%. This would be assumed to be a background level. Of 20 strains tested, 16 were inhibited at rates higher than 9%. Among these strains were 19 strains that had not been killed by the 4223 anti-M56  
25 r200 kDa antibody.

To summarize and as shown in Tables 1, 8 and 9, in our collection of 89 strains of *Moraxella catarrhalis*, 80 express 200 kDa. Of 57 strains tested with 4223 anti-M56 r200 kDa antibody in the bactericidal antibody assay, 39 were killed (58%). An additional 15 strains were inhibited from binding to epithelial cells by the  
30 same antibody for a total of 54 strains (95%), against which a single antibody was

effective. These data demonstrate the very high potential of r200 kDa proteins as vaccine antigens.

#### Example 13

This Example describes the sequence analysis of the 200 kDa protein gene from *M. catarrhalis* strain RH408, the non-clumping variant of 4223 described in WO 96/34960.

As described in Example 4 and Table 5, it appeared that the number of G nucleotides in the G tract had a regulatory function on the expression of the 200 kDa gene. *M. catarrhalis* strain 4223 and its non-clumping derivative RH408 appeared to differ only in the expression of the 200 kDa gene. The 200 kDa gene from strain RH408 was subcloned and sequenced and its sequence compared to the parental gene from strain 4223.

Four partially overlapping fragments of the 200 kDa protein gene were PCR amplified from strain *M. catarrhalis* RH408, using primers illustrated in Figure 16 and Table 10, under the conditions set out in Table 11. The combined sequences of the four PCR products covered approximately 6.5 kb including the entire 200 kDa protein gene and its flanking regions. When the sequence of the 6.5 kb fragment was compared with the sequence of the same region from its parent strain 4223, the only difference was the number of G nucleotides in the G tract. As described in Example 4, the correct number of G nucleotides in the G tract was nine. However, the number G nucleotides in the G tract of RH408 was only eight.

This result, along with the analysis of this region in 24 other strains of *M. catarrhalis* (Table 5) strongly suggests that the number of G nucleotides in the G tract controls the expression of the 200 kDa gene in *M. catarrhalis* strains. Similar mechanisms of transcriptional control are found for other bacterial genes, such as the *N. gonorrhoeae Pilc* gene (ref. 32).

#### Example 14

This Example describes the generation of additional N-terminal truncated r200 kDa proteins and expression studies.

As described in Example 6, the full-length r200 kDa protein appeared to be toxic to *E. coli* and could not be expressed under normal induction conditions. The M56 r200 kDa proteins were readily expressed, as described in Example 7, and were

subsequently shown to be highly promising vaccine candidates in *in vitro* assays (Examples 11 and 12). The expression of r200 kDa proteins of intermediate length and their properties was studied.

Three additional N-terminal truncated 200 kDa genes were constructed from the 4223 200 kDa gene using the procedures described in Example 7. The sites of truncation were chosen based upon and are illustrated in Figure 17. The arrows in Figure 17 indicate the sites of truncation, namely ALA<sup>12</sup>, VAL<sup>19</sup> and GLY<sup>39</sup>, each modified to MET. A 5' fragment up to an internal site was PCR amplified using primers illustrated in Table 8. For the ALA<sup>12</sup> truncation, the primers were 5' 6242.KS and 3' 4257.KS, for the VAL<sup>19</sup> truncation, the primers were 5' 6243.KS and 3' 4257.KS and for the GLY<sup>39</sup> truncation, the primers were 5' 6244.KS and 3' 4257.KS (Table 10). The amplification conditions were the same as those used for pKS348 (Table 11). The PCR products were restricted with *Nde*I and ligated into the *Nde*I sites of pKS348 for expression. While some expression of r200 kDa was obtained with each of the N-terminal truncations, the level did not approach the levels obtained using pKS348.

#### Example 15

This Example illustrates the construction of plasmids pQWE and pQWF expressing C-terminal fragments of the 200 kDa gene.

As shown in the amino acid comparison of Figure 6, the carboxy half of the 200 kDa protein is quite conserved, the main difference being a large approximately 300 amino acid residue insert in strain LES-1. Since so much cross-reactivity for the anti-M56 r200 kDa antisera had been observed, the conserved carboxy half of the protein was expressed.

Plasmid pKS348 prepared as described in Example 7 was digested with restriction enzymes, *Nde* I and *Nae* I, producing four fragments. The approximately 5.8 kb *Nde* I/*Nae* I fragment containing the T7 promoter, ampicillin antibiotic resistance marker and the 3' end of the 200 kDa gene was agarose gel purified. The approximately 480 bp *Nde* I/*Nde* I fragment containing the 5' end of the 200 kDa gene was also gel purified. This approximately 480 bp fragment was then restriction digested with the enzymes *Nla* IV and *Pst* I and the *Nde* I/*Nla* IV fragment ligated to the previously isolated 5.8 kb *Nde* I/*Nae* I fragment to produce plasmid pQWE, as

illustrated in Figure 19. This plasmid construct contained a 200 kDa gene with the *Nla* IV to *Nae* I fragment deleted. This plasmid construct resulted, upon expression as described in Example 7, in a fusion 200 kDa protein containing a very short piece of the 5' end and the 3' half of the 200 kDa protein.

5           An approximately 500 bp fragment around the *Eco* RI site in the 200 kDa gene from plasmid pKS348<sup>1</sup> was PCR amplified utilizing a 5' oligonucleotide, 6425.KS and a 3' oligonucleotide 4272.KS (Table 10) using the conditions outlined in Table 11. The 5' oligonucleotide was synthesized with an ATG translational start codon and a *Nde* I restriction site, while the 3' oligonucleotide was synthesized with  
10   an *Eco* RI site. The approximately 500 bp PCR fragment was the restriction digested with the enzymes *Nde* I and *Eco* RI. Plasmid pQWE, prepared as described above, was restriction digested with *Nde* I and *Eco* RI as illustrated in Figure 20, and this larger fragment agarose gel purified. The *Nde* I/*Eco* RI PCR fragment was then ligated into the isolated *Nde* I/*Eco* RI fragment from pQWE, to produce plasmid  
15   pQWF. This construct expresses a 5' truncated 200 kDa protein, having only the 3' half of this protein from the region about 40 bp upstream of the *Nde* I site to the 3' end.

          The constructs pQWE and pQWF, prepared as described above and as illustrated in Figures 19 and 20, were expressed in *E. coli* strain BL21(DE3)/pLysS  
20   as described in Example 7. The C-terminal half proteins were obtained at levels of expression approximately twice those achieved using pKS348. Corresponding constructs were prepared from strain LES-1 and produced comparable results.

          Antiserum was raised against the C-terminal half of 200 kDa protein produced from construct pQWE following the procedure of Example 10 and was  
25   employed in the bactericidal assay described in Example 11. As may be seen in Table 1B the antiserum showed more than 30% of killing against 30 out of 31 strains which were killed by the bactericidal assay using antiserum raised against the product from pKS348.

#### Example 16

30           This Example describes the identification of a natural site of C-terminal truncation for r200 kDa.

The full-length *M. catarrhalis* r200 kDa gene containing the putative signal sequence, could not be expressed in *E. coli*, as described above in Example 6. When the sequence encoding the first 56 amino acids was deleted, the M56 r200 kDa protein could be produced, but not in very high yield (plasmid pKS348), as described in Example 7. When the variable N-terminal half of the 200 kDa gene was completely deleted, a high expression level of the 3' r200 kDa protein was achieved (plasmid pQWF), as described in Example 15. By electrophoretic analysis, the 3' r200 kDa protein produced from pQWF was observed as two bands, with a significant portion of the product appearing as the lower molecular weight, presumed degradation product. The two 3' r200 kDa protein bands were purified and submitted to N-terminal and C-terminal sequence analyses. The N-terminal sequence was found to be identical for both bands and as expected. The C-terminal sequence of the higher molecular weight protein was as expected from the gene sequence, but the lower molecular weight protein did not have the expected C-terminal sequence. The possible C-terminal sequences were:

H V G A A K (SEQ ID No: 33)  
 . Y A V V . (SEQ ID No: 34)  
 . . V G G . (SEQ ID No: 35)  
 . . Q . . . (SEQ ID No: 36)  
 . . F . . . (SEQ ID No: 37)

An analysis of the 200 kDa gene (Figure 21) identified three possible sites of matching sequence: GVVK (SEQ ID No: 38) at approximately residue 400; VLGGK (SEQ ID No: 39) at approximately residue 1660; and VVAGK (SEQ ID No: 40) at approximately residue 1820 (see Figure 21). Of these, the first site does not appear in the 3' r200 kDa protein and only a truncation at the third site, the VVAGK sequence, would result in a protein of approximately the observed size.

#### Example 17

This Example describes the construction of a plasmid expressing a defined portion of the 200 kDa gene as illustrated in Figure 23.

Plasmid pQWF, prepared as described in Example 15, is a pT7-7 based plasmid containing the conserved 3'-half of the *M. catarrhalis* 200 kDa gene.

Plasmid pQWF was digested with *Dra* III and *Pst* I to remove 1.1 kb of the extreme 3'-end of the 200 kDa gene (Figure 23). A 260 bp PCR fragment was amplified, containing tandem stop codons after the VVAGK sequence. The PCR primers were designed to contain flanking *Dra* III and *Pst* I sites:

```

5           Dra III
      A S G K H S V A I G F
5' GCCTCAGGCAAG CACTCAGTG GCGATAGGTTTC           3' 7243.SL (SEQ ID No: 41)

      G T G N V V A G K * *
10 GGTACAGGCAATGTGGTAGCAGGTAAGTAATAGCTGCAGCCGG      (SEQ ID No: 44)
3' CCATGTCCGTTACACCATCGTCCATTATTATC GACGTC GGCC      (SEQ ID No: 43)
                                     Pst I
                                     5' 7244.SL (SEQ ID No: 42)

```

The 260 bp PCR fragment was digested with *Dra* III and *Pst* I and inserted into the digested pQWF, generating plasmids OA-54-1-6 and OA-54-2-7, pT7 3' 200 kDa(t), containing the carboxy-terminal truncated 3' 200 kDa gene (see Figure 22)(nucleotide sequence, SEQ ID No: 47; amino acid sequence, SEQ ID No: 48). In order to change the antibiotic selection from ampicillin to kanamycin, the *Bgl* II – *Pst* I T7 3' 200 kDa(t) gene cassette was excised and cloned into plasmid DS-1843-2, a pBR 328-based plasmid containing a multiple cloning site between the *EcoR* I and *Pst* I sites. The resulting pBR T7 3' 200 kDa(t) plasmid containing the C-terminally truncated 3' r200 kDa(t) was subsequently linearized with *Sal* I, dephosphorylated, and the 1.4 kb *Sal* I *kanR* gene from pUC-4K was inserted, generating pBR T7 3' 200 kDa (t)/*kanR*. Plasmid DS-2224-1-4 contains a synthetic *E. coli cer* locus that can be used to stabilize plasmids. pBR T7 3' 200 kDa(t) was linearized with *BamH* I, dephosphorylated, and the approximately 200 bp *BamH* I *cer* fragment from DS-2224-1-4 was inserted, generating pBR T7 3' 200 kDa(t)/*kanR/cer*.

#### Example 18

This Example illustrates the expression of 3' r200 kDa(t) from *E. coli*.

Plasmids OA-54-1-6 and OA-54-2-7, prepared as described in Example 17, were introduced by transformation into *E. coli* BL21(DE3) cells to generate strains OA-54-1-6-1/BL21(DE3), OA-54-1-6-2/BL21(DE3), OA-54-2-7-1/BL21(DE3) and OA-54-2-7-2/BL21(DE3). Plasmids OA-54-1-6 and OA-54-2-7 were introduced by transformation into *E. coli* BL21-SI cells (Life Technologies) to generate strains OA-54-1-6-1/BL21-SI, OA-54-1-6-2/BL21-SI, OA-54-2-7-1/BL21-SI, and OA-54-



2-7-2/BL21-SI. BL21(DE3) strains were grown to OD  $A_{578}$  of 0.4 in NZCY medium, and induced with 0.4mM IPTG for 4 hours. BL-21-SI strains were grown to OD  $A_{578}$  of 0.3 to 0.4 in LBON (Luria broth without NaCl) medium and induced with 0.3M sodium chloride for 4 hours. Cell lysates were analysed on 8% SDS-PAGE (Figure 3). Duplicate strains were found to be equivalent and the expression from both hosts was about 10 to 20% of total protein.

#### SUMMARY OF THE DISCLOSURE

In summary of this disclosure, nucleotide sequences encoding an about 200 kDa outer membrane protein from several strains of *Moraxella catarrhalis* are described along with recombinant production of such protein. Modifications are possible within the scope of this invention.

**Table 1A**Examination of 200 kDa protein in *M. catarrhalis* strains

STRAIN	ANATOMICAL ORIGIN	SOURCE	EXPRESSION OF 200 kDa PROTEIN
4223	MID. EAR FLUID	T.F. MURPHY	+++
RH408	MUTANT OF 4223		-
3	SPUTUM	"	-
56	SPUTUM	"	-
135	MID. EAR FLUID	"	+++
585	BACTEREMIA	"	+
5191	MID. EAR FLUID	"	+++
8185	NASOPHARYNX	"	+++
M2	SPUTUM	"	+++
M5	SPUTUM	"	-
ATCC25240		ATCC	-
H-04	OTTIS	G.D. CAMPBELL	+++
H-12	"	"	-
PO-34	"	"	+++
PO-51	"	"	+++
E-07	"	"	+++
E-22	"	"	+++
E-23	"	"	+++
E-24	"	"	+++
M-02	"	"	+++
M-20	"	"	+++
M-29	"	"	+++
M-32	"	"	+++
M-35	"	"	+++
Q-2	EXPECTORATION	M.G. BERGERON	+
Q-6	"	"	-
Q-8	"	"	+++
Q-9	"	"	-
Q-10	"	"	+++
Q-11	"	"	+++
Q-12	"	"	-
R-1	BRONCHIAL SECRETIONS	"	+
R-2	"	"	-
R-4	OTTIS	"	+++
R-5	"	"	+++
R-6	"	"	+++
R-7	"	"	+++
N-209	BLOOD	"	+++
VH-1	OTTIS	V. HOWIE	+++
VH-2	"	"	+++
VH-3	"	"	+++
VH-4	"	"	+++
VH-5	"	"	+++
VH-6	"	"	+++
VH-7	"	"	+++

VH-8	"	"	+++
VH-9	"	"	+++
VH-10	"	"	+++
VH-11	"	"	+++
VH-12	"	"	+++
VH-13	"	"	+++
VH-14	"	"	+++
VH-15	"	"	+++
VH-16	"	"	+++
VH-17	"	"	+++
VH-18	"	"	+++
VH-19	"	"	+++
VH-20	"	"	+++
VH-23	"	"	+++
VH-24	"	"	+++
VH-25	"	"	+++
VH-26	"	"	+++
VH-27	"	"	+++
VH-28	"	"	+++
VH-29	"	"	+++
VH-30	"	"	+++
LES1	OTITIS	L.S. STENFORS	+++
LES2	"	"	+++
LES4	"	"	+++
LES5	"	"	+++
LES6	"	"	+++
LES7	"	"	+++
LES8	"	"	+++
LES9	"	"	+++
LES10	"	"	+++
LES11	"	"	+++
LES12	"	"	+++
LES13	"	"	+++
LES16	"	"	+++
LES17	"	"	+++
LES21	"	"	+++
30607	OTITIS	C.W. FORD	+++
CJ1	"	C. JOHNSON	+++
CJ3	"	"	+++
CJ4	"	"	+++
CJ7	"	"	+++
CJ8	"	"	+++
CJ9	"	"	+++
CJ11	"	"	+++

Bacteria were lysed and proteins were separated on SDS-PAGE gels. The expression of 200 kDa protein was examined by Coomassie Blue staining and by Western blot using anti-200 kDa protein guinea pig serum.

**TABLE 1B**

Bactericidal assay results against *Moraxella catarrhalis* using antisera raised against recombinant M56 200 kDa protein from strains 4223 and LES1, and recombinant C-terminal half of 200 kDa protein from strain 4223.

STRAIN	Killed by anti-M56 200 kDa from 4223	Killed by anti-C- terminal half of 200 kDa from 4223	Killed by anti-M56 200 kDa from LES1
4223	++	++	-
135	++	++	++
H-04	++	++	?
H-12*	-	NT	-
PO-34	-	NT	++
PO-51	-	NT	-
E-07	-	NT	++
E-22	++	++	-
E-24	-	NT	-
M-02	++	++	++
M-20	++	+	-
M-29	++	++	++
M-32	++	++	++
M-35	++	++	++
R4	-	NT	++
R5	++	++	++
R6	++	+	+
R7	++	NT	?
Q8**	++	+	NT
VH-1	++	NT	++
VH-2	++	NT	++
VH-4	-	NT	++
VH-5	++	++	-
VH-7	++	+	?
VH-8	++	++	++
VH-9	-	NT	++
VH-10	++	++	++
VH-13	-	NT	-
VH-15	++	++	++
VH-17	-	NT	-
VH-19	++	++	++
VH-20	+	+	++
VH-23	+	NT	++
VH-24	++	++	-
VH-25	-	NT	++
VH-26	-	NT	++
VH-27	-	NT	-
VH-28	+	NT	-
VH-29	++	++	++
VH-30	-	NT	++
LES1	-	NT	++
LES2	++	++	+
LES4	+	NT	++
LES5	-	NT	++

LES9	++	++	++
LES11	+	+	+
LES12	-	NT	?
LES13	-	NT	++
LES16	+	++	++
LES17	++	++	-
LES21	++	++	-
30607	+	NT	++
CJ1	++	++	++
CJ3	++	-	++
CJ4	++	++	++
CJ7	++	++	++
CJ8	++	++	?

\* This strain does not produce 200 kDa protein.

\*\* This is the only non-otitis media strain (isolated from expectorate) in this Table.

++: Killed more than 60% (>60%), +: killed between 30% and 60%,

-: killed 30% or less, NT: not tested, ?: the results not tested.

**TABLE 2**

The number of G nucleotides in the G tract of the 200 kDa protein gene determined by sequencing of subcloned genes from a  $\lambda$ EMBL3 clone.

Plasmid*	Number of G's
pKS10	10
pKS59	10
PKS63	10
PKS71	10

\* pKS10 and pKS71 carried a DNA insert directly subcloned from a  $\lambda$ EMBL3 clone. pKS59 and pKS63 carried a subcloned DNA fragment, pKS9, which was a subclone from an  $\lambda$ EMBL3 clone. pKS59, pKS63 and pKS71 carried identical DNA inserts.

**TABLE 3**

The number of G nucleotides in the G tract of the 200 kDa protein gene amplified by PCR from subcloned genes

Primers	Template DNA*	Number of G's
4211 and 4213	pKS9	10
4211 and 4213	pKS10	10
4211 and 4213	pKS71	10

\* pKS9, pKS10 and pKS71, which contain a 5' fragment of the 200 kDa protein gene, were independently subcloned from the  $\lambda$ EMBL3 clone.

**TABLE 4**

The number of G nucleotides in the G tract of the 200 kDa protein gene amplified by PCR from chromosomal DNA of strain 4223

Primers	Template*	Number of G
4211 and 4166	4223B	9
4211 and 4213	4223B	9
4211 and 4213	4223R	9

\* The template chromosomal DNAs, 4223B and 4223R, were independently prepared from *M. catarrhalis* strain 4223.

**TABLE 5**

The number of G nucleotides in the G tract in different strains of *M. catarrhalis*

Expression	Number of G	Number of strains examined	Possible start codon
+++	3	1	ATG
+++	6	7	ATG
+++	9	7	ATG
+	10	3	GTG
-	7	3	GTG
-	8	2	GTG
-	9	1*	ATG
Total		24	

\* The 200 kDa protein gene of this strain was prematurely terminated by a stop codon.

**TABLE 6****Anti-M56 r200 kDa antibody titers in guinea pig and rabbit sera**

ANTISERA	ANTIBODY TITERS	
	Against M56 r200 kDa (4223)	Against M56 r200 kDa (LES-1)
Gp anti-r200 kDa (4223)	204,800 409,600	102,400 409,600
Gp anti-r200 kDa (LES1)	204,800 102,400	1,638,400 1,638,400
Rb anti-r200 kDa (4223)	102,400 102,400	102,400 102,400
Rb anti-r200 kDa (LES1)	25,600 102,400	204,800 409,600



TABLE 7

Killing of *M. catarrhalis* strain 4223 by the bactericidal antibody activity of guinea pig anti-M56 r200 kDa protein serum

Serum dilution	1/64	1/128	1/256	1/512	1/1024
Killing %	97%	95%	95%	80%	38%

\* The guinea pig antiserum was raised against M56 r200 kDa protein from strain 4223, and the bactericidal antibody activity of the serum at various dilutions were examined against the strain 4223.

TABLE 8

Inhibition of the binding of *M. catarrhalis* strains to Chang cells by guinea pig anti-M56 r200 kDa protein serum

Strain	1/4	1/16	1/64
4223	98%	92%	83%
Q8	77%	82%	55%

\* The guinea pig antiserum was raised against M56 r200 kDa protein from strain 4223.

**TABLE 9**

Inhibition of *in vitro* adherence of *Moraxella catarrhalis* to Hep-2 cells by antiserum raised against recombinant 200 kDa protein from strain 4223

STRAIN	Inhibition
4223*	+++
PO-34	+++
PO-51	++
E-07	++
R4	++
VH-4	++
VH-9	-
VH-13	+
VH-17	++
VH-23	++
VH-25	++
VH-26	+++
VH-27	+
VH-28	+++
LES1	++
LES4	-
LES12	-
LES13	-
30607	+

+++ : Inhibition was 30% or higher, ++ : Inhibition was 20% to 30%, + : Inhibition was 15% to 20%, - : Inhibition was lower than 15%.

\*: This strain is the positive control, and the only strain in this Table which was killed by the bactericidal activity of anti-recombinant 200 kDa protein serum.

TABLE 10

Nucleotide sequences of primers used for PCR amplifications

PRIMER	NUCLEOTIDE SEQUENCE	SEQ ID No:
4211.KS	GATGCCTACGAGTTGATTTGGGT	14
4213.KS	GAGCGTTGCACCGATCACGAGGA	15
4166.KS	CACTAGCCTTTACATCACCACCGATG	16
5295.KS	AAGGTAAACCCATATGAATCACATCTATAAAGTCA	17
4260.KS	GCTTCTAGCTGTGCCACATTGA	18
5471.KS	CGCTCGCTGTCCATATGATCGGTGCAACGCTCA	19
4257.KS	GACCCTGTGCATATGACATGGCT	20
4254.KS	CCTTGGCATCAATCGTGGCACA	21
4278.KS	TTACCTGCATCAATGCCATTGTCT	22
4329.KS	CTGAGGTGAATACAACTACA	23
4272.KS	CATCAGAGGTCTTTGAGGTGTCAT	24
4118.KS	CATCACCGTGGGTCAAAGAACGCA	25
4267.KS	GATGTCGGCAATGTTTACCTGA	26
4269.KS	CCACATTGACCAGTACTGGCACAGGTGCTA	27
4981.KS	ACCTATGATCAATGGCGATTTGGT	28
6425.KS	AAAGATCATATGGTTACCTTTGGCATTAAAC	29
6242	GTCATCTTTCATATGGCCACAGGCACA	30
6243	ACATTTATGCATATGGCAGAGTACGCCA	31
6244	GCTACAGGGCATATGGGCAGTGTATGCACT	32

TABLE 11PCR Cycle Conditions

1. For the construction of pKS294, oligonucleotides 5295 and 4260 and of pKS348, oligonucleotides 5471 and 4257:  
95°C for 2 min → 95°C for 1 min, 60°C for 30 sec, 72°C for 1 min (10 cycles) → 95°C for 1 min, 62°C for 30 sec, 72°C for 1 min (20 cycles with extension of 1 sec/cycle) → 72°C for 10 min → 4°C.
2. For the construction of pQWF, oligonucleotides 6425 and 4272:  
95°C for 2 min → 95°C for 1 min, 60°C for 30 sec, 72°C for 1 min (10 cycles) → 95°C for 1 min, 60°C for 30 sec, 72°C for 1 min (20 cycles with extension of 1 sec/cycle) → 72°C for 10 min → 4°C.
3. For the amplification of 700 bp fragment for sequencing the G-nucleotide tract from different strains, oligonucleotides 4211 and 4166.  
95°C for 2 min → 95°C for 1 min, 60°C for 1 min, 72°C for 2 min (10 cycles) → 95°C for 1 min, 60°C for 1 min, 72°C for 2 min (20 cycles with extension of 5 sec/cycle) → 72°C for 10 min → 4°C.
4. For sequencing 200 kDa protein from *M. catarrhalis* strain RH408,  
(a) oligonucleotides 4254 and 4278; 4118 and 4267; and 4269 and 4981:  
95°C for 2 min → 95°C for 1 min, 62°C for 30 sec, 72°C for 1 min (10 cycles) → 95°C for 1 min, 62°C for 30 sec, 72°C for 1 min (20 cycles with extension of 2 sec/cycle) → 72°C for 10 min → 4°C.  
(b) oligonucleotides 4329 and 4272  
95°C for 2 min → 95°C for 1 min, 58°C for 30 sec, 72°C for 1 min 30 sec (10 cycles) → 95°C for 1 min, 58°C for 30 sec, 72°C for 1 min 30 sec (20 cycles with extension of 1 sec/cycle) → 72°C for 10 min → 4°C.

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